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THE HISTOPATHOLOGY OF FIBROUS DYSPLASIA OF BONE IN PATIENTS WITH ACTIVATING MUTATIONS OF THE Gs*α* GENE: SITE-SPECIFIC PATTERNS AND RECURRENT HISTOLOGICAL HALLMARKS

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SUMMARY

Gs*α* mutations and histopathology have been analysed in a series of 13 patients with fibrous dysplasia (FD) of bone, including 12 patients with the McCune–Albright syndrome (MAS) and one patient with monostotic FD. Activating mutations (either R201C or R201H) of the gene encoding the *α* subunit of the stimulatory G protein, Gs, were detected in all cases, including the case of monostotic FD, using a variety of techniques [reverse transcription-polymerase chain reaction (RT-PCR) with allele-specific primers, allele-specific oligonucleotide hybridization, and DNA sequencing]. A spectrum of bone lesions associated with such mutations was identified and it was possible to recognize three primary, but distinct, histological patterns, defined here as Chinese writing type, sclerotic/Pagetoid type, and sclerotic/hypercellular type, which are characteristically associated with the axial/appendicular skeleton, cranial bones, or gnathic bones, respectively. Features of FD histopathology were characterized by confocal fluorescence microscopy, which allowed the definition of osteogenic cell shape changes and 'Sharpey fibre bone' as common denominators of all histological subtypes. Defining characteristics of the different subtypes, two of which diverge from standard descriptions of FD and have never been characterized before, were dependent on the amount and structure of bone tissue within the FD lesion. These data emphasize the non-random (site-specific) variability of FD histopathology in patients carrying activating mutations of the Gs*α* gene and provide additional evidence for the occurrence of Gs*α* mutations in cases of FD other than typical MAS. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS—**bone; McCune–Albright syndrome; fibrous dysplasia; G protein; Gs*α* mutation; pathology; confocal microscopy**

INTRODUCTION

Fibrous dysplasia (FD) of bone is a common, crippling skeletal disorder, usually appearing in childhood or adolescence,[1–3] which leads to severe consequences including pathological fractures, impairment of limb function, facial and limb deformities, and compressive damage of sensory nerves resulting in blindness or deafness. Most commonly, FD is described as the replacement of normal bone and marrow by an abnormal fibrous tissue within which irregular trabeculae of woven bone are haphazardly distributed. On clinical grounds, three forms of the disease are usually distinguished: a monostotic form, presenting in early adolescence as a single localized bone lesion, typically in a femur or a cranial bone; a polyostotic form, typically presenting in late childhood and affecting multiple skeletal sites; and a polyostotic form associated with endocrinopathies and skin pigmentation, also known as the McCune–Albright syndrome (MAS).[4,5] The discovery of activating missense mutations of the gene encoding the *α* subunit of the stimulatory G protein, Gs, in patients with MAS[6,7] has paved the way towards an understanding of the cellular and molecular mechanisms underlying the development of FD in bone. Gs*α* mutations have been demonstrated in the affected bones[8–11] and in cells grown from bone lesions.[12–14] Recent data indicate that histological features observed in FD of MAS patients can indeed be seen as the expression of dysregulation of osteogenic cells related to increased levels of cAMP, which are in turn predicted from activating Gs*α* mutation and the resultant overactivity of adenylyl cyclase.[15]

In this study, the pathology of bone lesions observed in a series of 13 patients with FD of bone was analysed by histology and confocal microscopy, along with an analysis of Gs*α* mutations. The goal of the study was to redefine the histopathology of FD of bone in patients who have proven mutations of the Gs*α* gene. We identified diverse histological patterns of bone lesions associated with different anatomical sites. We also

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Table I—Summary of clinical data and mutation analysis

Patient	Sex/age (years)	Clinical presentation	Gsα mutation	Site of bone lesions	References
1	F10	MAS	R201H	g*	15, pt 5
2	M25	MAS	R201C	c	
3	F10	MFD	R201H	c	
4	F7	MAS	R201H	g, f	15, pt 4
5	F6	MAS	R201H	f	15, pt 6
6	F4 mo	MAS	R201C	v, r, f	
7	M17	MAS	R201C	r, h, f, c	6, pt 2; 15, pt 1
8	M5	MAS	R201H	r, v	6, pt 3
9	F7	MAS	R201H	f	8, pt 2
10	F7	MAS	R201H	f	8, pt 3
11	M3	MAS	R201C	f	18
12	F29	MAS	R201H	f	19, pt 1
13	M7	MAS	R201C	f	16

pt=Patient; M, F=male, female; mo=months; MAS=McCune–Albright syndrome; MFD=monostotic fibrous dysplasia of bone; g=gnathic bones; c=cranial bones; f=femur; v=vertebra; r=rib; h=humerus; R201H=Arg to His; R201C=Arg to Cys.
*Two separate lesions.

characterized a few key elementary lesions, likely related to the effects of mutation on osteogenic cells, which represent a common denominator of the diverse patterns. Finally, we obtained evidence for the occurrence, in a case of monostotic FD, of a mutation of the Gsα gene of the same type as those characterizing MAS.

MATERIALS AND METHODS

Patients and tissues

A synopsis of patient data and pathological material used for this study is provided in Table I.

Fresh samples of bone lesions from cases 1–3 were collected under an NIH IRB approved protocol (97-DK-0055). Cases 1 and 2 were from patients with complete clinical features of MAS, whereas patient 3 underwent surgery for a single lesion of the right temporal bone, which was radiologically consistent with FD, but in the absence of endocrine abnormalities or skin pigmentation. Paraffin blocks from these samples were prepared following decalcification in neutral EDTA. Archival paraffin blocks or histological slides were available for cases 4–13. Clinical data for cases 7–10 have been reported elsewhere.[6,8,16–18]

Mutation analysis

Details of the establishment of stromal cell cultures from samples of affected and normal bone (derived from normal volunteers under an NIH IRB approved protocol, 94-D-0188) have been reported previously.[19] Gsα mutations were demonstrated by a PCR approach[8] in case 2, an RT-PCR approach[19] in cases 1 and 2, and by DNA sequencing in case 3. RNA from the affected and normal cultures was prepared by extraction with STAT 60™ according to the manufacturer's recommendations (TEL-TEST B, Inc., Friendswood, TX, U.S.A.) and used for RT-PCR. A ~300 bp sequence in the α subunit cDNA was chosen as the target for PCR amplification. Three 5′ oligonucleotide primers were designed based on the normal and mutated sequences of exon 8 of the Gsα gene. An additional single base mismatch towards the 3′ end was included to ensure that the normal mRNA was not primed by the mutated sequence. One single reverse primer complementary to a sequence of exon 11 was used in all the reactions (GenBank Accession number X04408).[20] The RT and PCR conditions were essentially as reported elsewhere.[15] The primers used are reported in Table II.

DNA from stromal cells and bone was extracted by DNA NOW™ according to the manufacturer's specification. A 270 bp sequence of the Gsα gene containing the codon for Arg 201 (GenBank Accession number M21142[21]) was amplified by PCR (primers used reported in Table II). The amplified product was purified by a Wizard PCR PREP DNA Purification System (Promega) and sequenced by the dideoxy chain-termination methods using an ABI 370 Automated DNA Sequencer and an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer).

DNA was extracted from a blood sample from case 4, and from available archival paraffin blocks of affected

Table II—Primers used in the RT-PCR and PCR reactions

RT-PCR (allele-specific primers)	
Wild-type	5′-GACCTGCTTCGCTGG*CG
Arg→His	5′-GGACCTGCTTCGCTGG*C(A)
Arg→Cys	5′-CAGGACCTGCTTCGCTC*C(T)
Reverse	5′-TCTTGCTTGTTGAGGAACAG
PCR	
5′-272-TGACTATGTGCCGAGCGA-289 (forward, exon 7)	
5′-521-AACCATGATCTCTGTTATATAA-542 (reverse, intron G)	

An asterisk denotes internal mismatching and parentheses denote the base transition.

tissues as described from cases 5–6 and 7–10.[6] Gsα mutations were demonstrated by allele-specific oligonucleotide hybridization (ASO) on samples of PCR-amplified genomic DNA as described previously.[6] Mutation data on cases 1 and 7–10 have been previously published.[6,8,15]

Confocal microscopy

Haematoxylin and eosin (H&E)-stained sections were studied by confocal fluorescence microscopy, taking advantage of the known properties of eosin as a fluorochrome[22] in order to investigate the fine detail of histological changes observed in standard diagnostic material. Single focal plane confocal images or stacks of images spanning the entire section thickness were recorded and reconstructed in a Sarastro Laser Scanning Confocal System equipped with a 488 argon ion laser and complemented with the Molecular Dynamics Image Space software run on a Silicon Graphics workstation.

RESULTS

Mutation analysis

Table I summarizes the results of mutation analysis in our series. The R201C mutation occurred in five cases and the R201H in eight; 12 cases out of 13 in our series represents patients with the complete triad of McCune–Albright syndrome (endocrinopathy, skin hyperpigmentation, and FD of bone) (Fig. 1A). Case 3, in contrast, does not present with the complete McCune–Albright phenotype (due to the lack of endocrinopathies and skin pigmentation) and represents an instance of Gsα mutation-associated, monostotic FD of the temporal bone (Fig. 1B).

Major histological patterns

Three main histopathological patterns of FD were observed in our series (Fig. 2). The three patterns were associated with different sites of the skeleton and differed from each other in the overall amount, architecture, and cellularity of the lesional bone tissue.

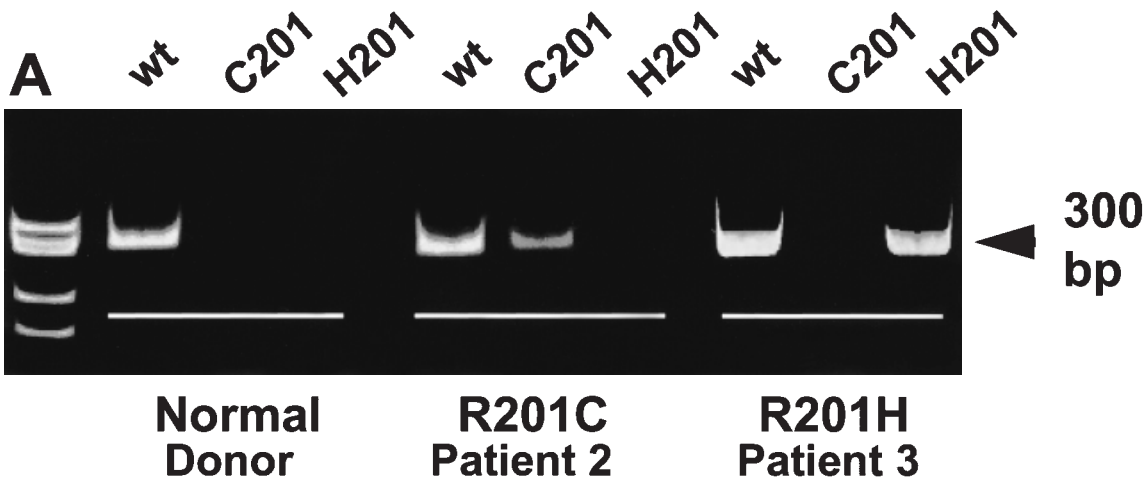
'Chinese writing' fibrous dysplasia—This pattern, corresponding to the best-known histology of FD, was observed in 15 samples from ten patients, namely in all lesions from limb bones and from the axial skeleton (rib, vertebrae), to the exclusion of craniofacial bones. The typical 'Chinese writing' pattern of bone trabeculae was the dominant feature. The bone trabeculae were thin and disconnected. Epithelial-like arrays of typical active osteoblasts were rarely seen. Active bone resorption was indicated by typical osteoclasts occurring singly. Resorption of the interior of bone trabeculae (so-called dissecting resorption), similar to the type observed in hyperparathyroidism-related trabecular bone lesions, was frequent. The fibrous tissue separating bone trabeculae was loose in the proximity of bone surfaces and denser towards the centre of the intertrabecular spaces. Most bone trabeculae were non-lamellar in texture, as revealed by polarized light microscopy. Prominent numbers of Sharpey fibres were observed over the trabecular profile (Fig. 3). Osteogenic cells associated with trabecular surfaces were noted for their retracted, stellate morphology (Fig. 3).

Pagetoid fibrous dysplasia—This pattern was consistently observed in all three cases of FD of cranial bones, including the single case of monostotic, Gsα mutation-positive FD in this series. Instead of thin, disconnected trabeculae taking up a small proportion of a largely fibrous background, a dense, sclerotic trabecular bone was observed in these cases. Overall, the appearance was closely reminiscent of a Paget-like pattern. This was further suggested by the occurrence of a rich system of cement/arrest lines closely resembling Schmorl's mosaic seen in Paget's disease of bone (Fig. 4). Bone trabeculae formed an uninterrupted network outlining fibrotic areas reminiscent of enlarged, abnormal Haversian canals. Systems of Sharpey fibres and stellate/retracted osteoblasts were easily appreciated along the trabecular surfaces (Fig. 4). The collagen texture of bone trabeculae was non-lamellar throughout.

Hypercellular fibrous dysplasia—This pattern was consistently observed in all three lesions observed in two cases of FD of gnathic bones. Overall, the pattern was characterized by the presence of significant amounts of bone, like the Pagetoid variant. However, bone trabeculae were discontinuous and distributed in a remarkably ordered, often parallel, pattern. Typically, one side of each trabecula was associated with readily recognized osteoblasts, sometimes arranged in multiple layers. The osteoblastic activity was polarized to one side of each trabecula, and to the homologous sides of parallel trabeculae. Deep to the osteoblastic layer, large clefts accommodating multiple developing osteocytes were seen (Fig. 5). The collagen texture was woven throughout the trabeculae. The soft tissue adjacent to the bony trabeculae was loose and consisted of stellate osteoblasts noted for elongated branches and processes of their cytoplasm. Sharpey fibres and retracted osteoblasts were obvious at sites of early bone formation (Fig. 5).

Other histological findings

In addition to the three main histological patterns, other findings included microscopic changes in grossly unaffected bones, growth plate anomalies, and the occurrence of bone lesions other than FD. In some samples free from overt changes of FD, microscopic foci recapitulating FD features were detected. The smallest lesions consisted essentially of an endosteal layer of elongated fibroblast-like cells, producing a pattern entirely similar to 'endosteal fibrosis' of hyperparathyroidism-related bone lesions. These areas of 'endosteal fibrosis' were free from haematopoietic tissue and from marrow adipocytes. Larger, but still microscopic lesional foci featured, in addition, small trabeculae of woven bone.



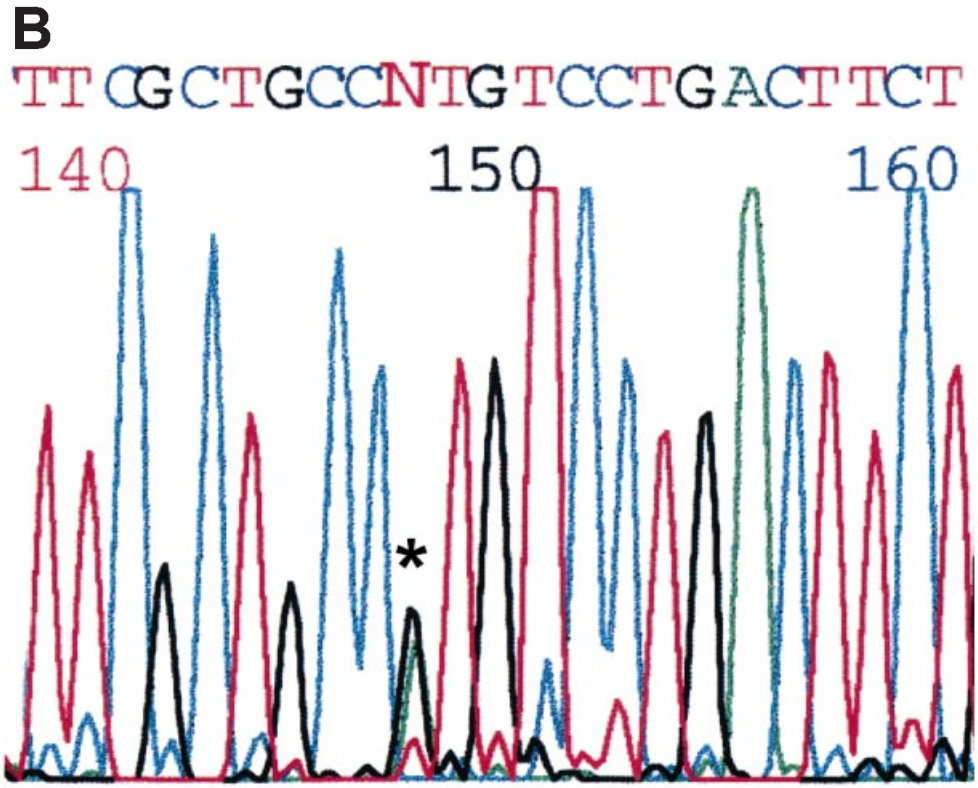


Fig. 1—(A) RT-PCR amplification using allele-specific primer sets described previously[19] predicts the generation of an amplification product of ~300 bp. Using mRNA from normal stromal cultures, there was amplification with only the wild-type primer set and no amplification with either of the mutated primer sets. Amplification of the wild-type allele was found in mRNA from all patients, consistent with the fact that mutant cells contain one normal allele. Either the R201H (case 1) or the R201C (case 2) mutations are specifically detected by the appropriate primer sets in individual patients. (B) DNA sequencing of PCR-amplified DNA isolated from a frozen bone specimen (monostotic FD), demonstrating the heterozygous G to A mutation encoding R201H

In four cases, bone lesions other than FD were identified in addition to lesions falling into one of the three main histological patterns described above ('Chinese writing', pagetoid, and hypercellular FD). These included a giant cell lesion closely reminiscent of a giant cell tumour, aneurysmal bone cysts observed in the maxilla of two different patients, and an angiomatoid lesion of a vertebra.

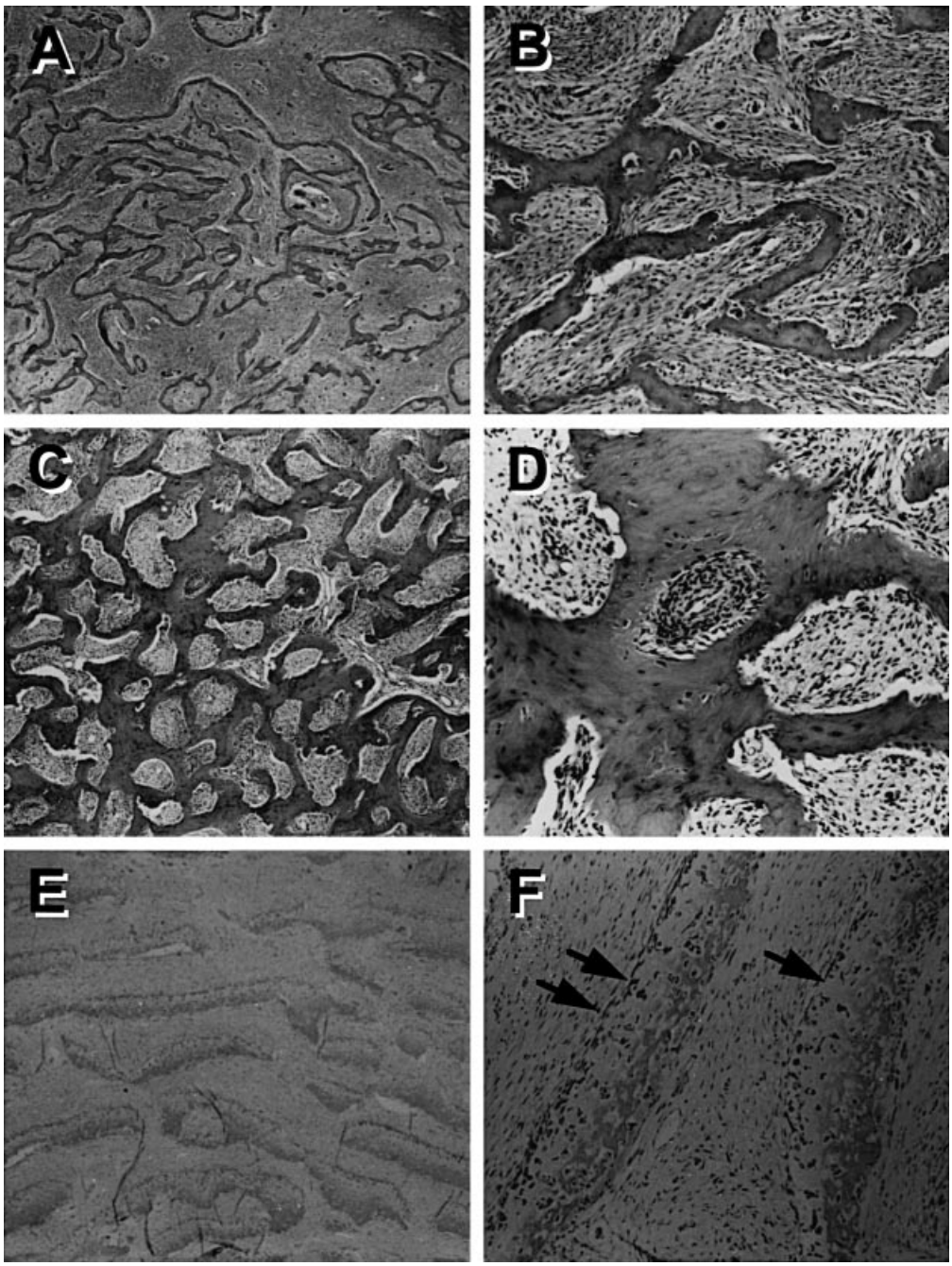


Fig. 2—Overview of the histology of the three main variants of R201C/R201H-associated FD of bone. (A, B) 'Chinese writing' pattern. Fibrous tissue predominates over thin, irregular, and disconnected trabeculae. (C, D) 'Pagetoid' FD of cranial bones. Trabeculae are thick and interconnected, and bone predominates over fibrous tissue. (E, F) 'Hypercellular' FD of gnathic bones. Substantial amounts of bone are arranged in conspicuous trabeculae noted for their parallel arrangement, for obvious osteoblastic layers (F, arrows), and for unusually numerous osteocytes. The osteoblastic layers in different trabeculae are polarized to homologous sides

DISCUSSION

This study indicates that activating mutations of the Gsα gene associated with MAS underlie a spectrum of bone lesions rather than a uniform histopathological pattern. Lesions whose morphology in fact adheres to the commonplace descriptions of FD of bone are essentially found in the metaphysis of long bones, in vertebrae, and in ribs. In contrast, lesions developing in the context of craniofacial bones are hardly consistent with such conventional descriptions. While it has long been known that FD of craniofacial bones produces radiologically hyperdense lesions,[2,23,24] a formal comparison of the histology of these lesions with those occurring elsewhere has never been attempted and little if any attention has been paid to the histology of such 'sclerotic' lesions. The recent development of medical treatment protocols for FD using bisphosphonates[25,26]

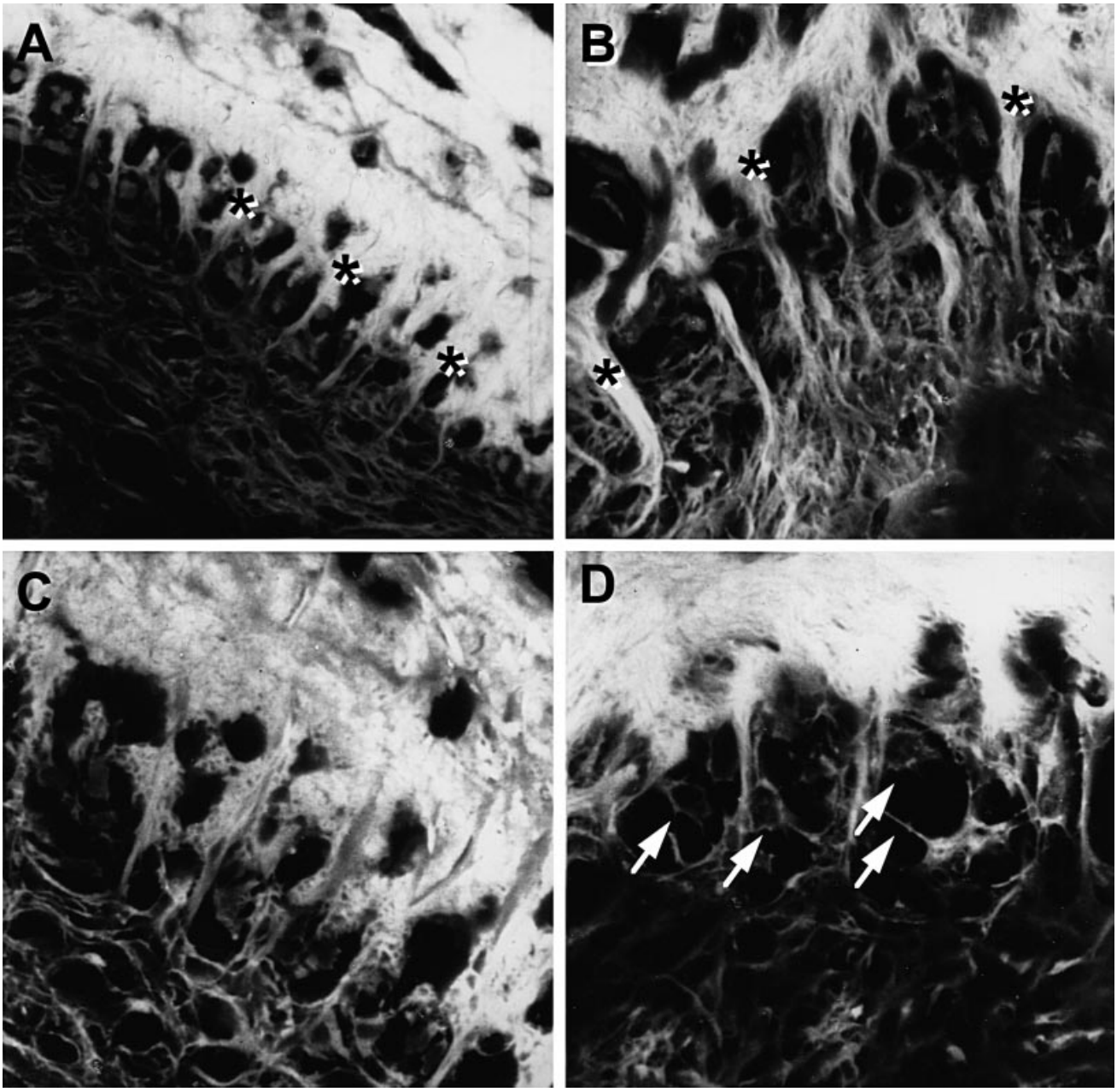


Fig. 3—Structural details of the 'Chinese writing' type of FD as imaged in confocal fluorescence microscopy of H&E sections. Regular arrays of abundant Sharpey fibres (bundles of collagen oriented perpendicular to the bone surface) (A and B, asterisks) are noted over the trabecular profile. Osteoblastic cells associated with these systems are noted for multiple thin cell processes resulting in a stellate/retracted cell shape (D, arrows)

makes an acquaintance with these lesions especially desirable. Since bisphosphonates increase bone mass mainly by reducing bone resorption, the impact of such treatments on the histology of 'sclerotic' variants of FD will require proper evaluation. We have identified and histologically characterized two distinct variants associated with non-gnathic cranial bones and gnathic bones, respectively. The two variants share a substantial proportion of lesional bone (a 'sclerotic' appearance) but otherwise diverge substantially in the histologic detail and are readily distinguished from one another and from the typical 'Chinese writing' pattern generally taken as the conventional description of FD. We termed one variant 'Pagetoid' based on the resemblance of the overall histological picture to Pagetic bone, as well as on the occurrence of a mosaic-like pattern of cement lines. Interestingly, 'Pagetoid' FD of cranial bones is a recognized radiographic appearance of these lesions,[2,23] which further encourages the introduction of this term to denote a distinct histological pattern. The other variant (hypercellular) appears to occur characteristically in gnathic bones and its histological detail has not been noted previously. We observed the same pattern in an additional case of MAS which was not included in the present series, due to the lack of samples suitable for mutation analysis. This pattern is, at a glance, not

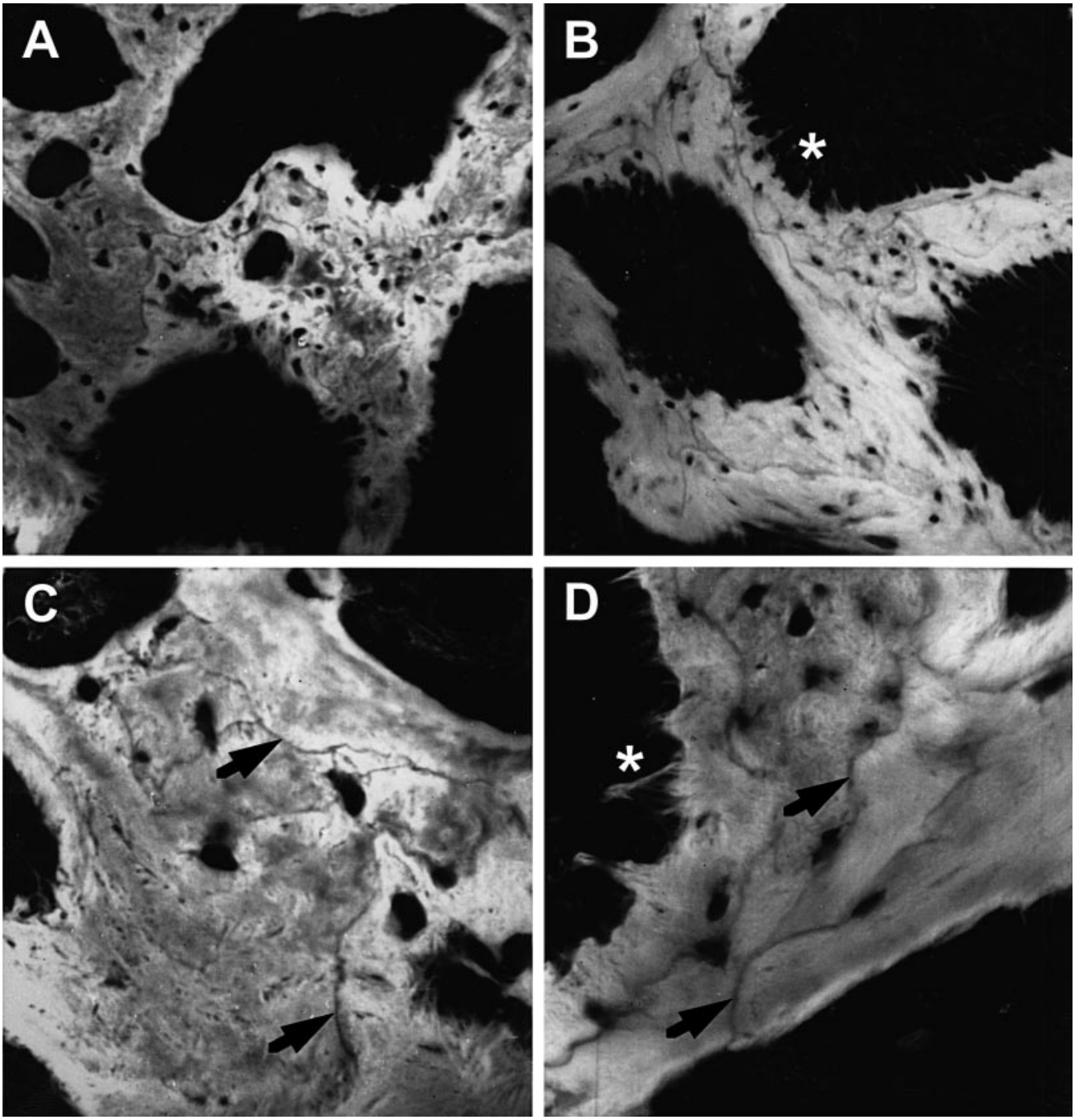


Fig. 4—Confocal images of the 'Pagetoid' variant of FD. Complex systems of arrest/reversal (cement) lines, reminiscent of Schmorl's mosaic of Paget's disease of bone, are visualized as linear non-fluorescent features within the bone matrix (C and D, arrows). Sharpey fibres impart a fuzzy profile to trabecular surfaces (B and D, asterisks)

reminiscent of conventional FD, due to the extreme abundance, rather than paucity, of readily recognized osteoblastic cells. The mutual arrangement of these cells and their position with respect to the trabecular geometry are further elements of singularity of this pattern.

The diverse histological patterns of FD are specifically associated with defined anatomical sites. Major differences in macro- and micro-anatomical features among the different skeletal sites can be reflected in the resultant histological diversity of the bone lesions. For example, the sclerotic attitude exhibited by craniofacial lesions may reflect the higher ratio of compact to cancellous bone at those sites, compared with the predominantly cancellous structure of the femur metaphysis or the vertebrae. This implies that the local bone environment may critically condition the pathological expression of Gsα mutations impacting on the osteogenic cell lineage. Alternatively, the different embryological origin of craniofacial bone (neuroectodermal rather than mesodermal) may also be invoked as a potential determinant of histological diversity.

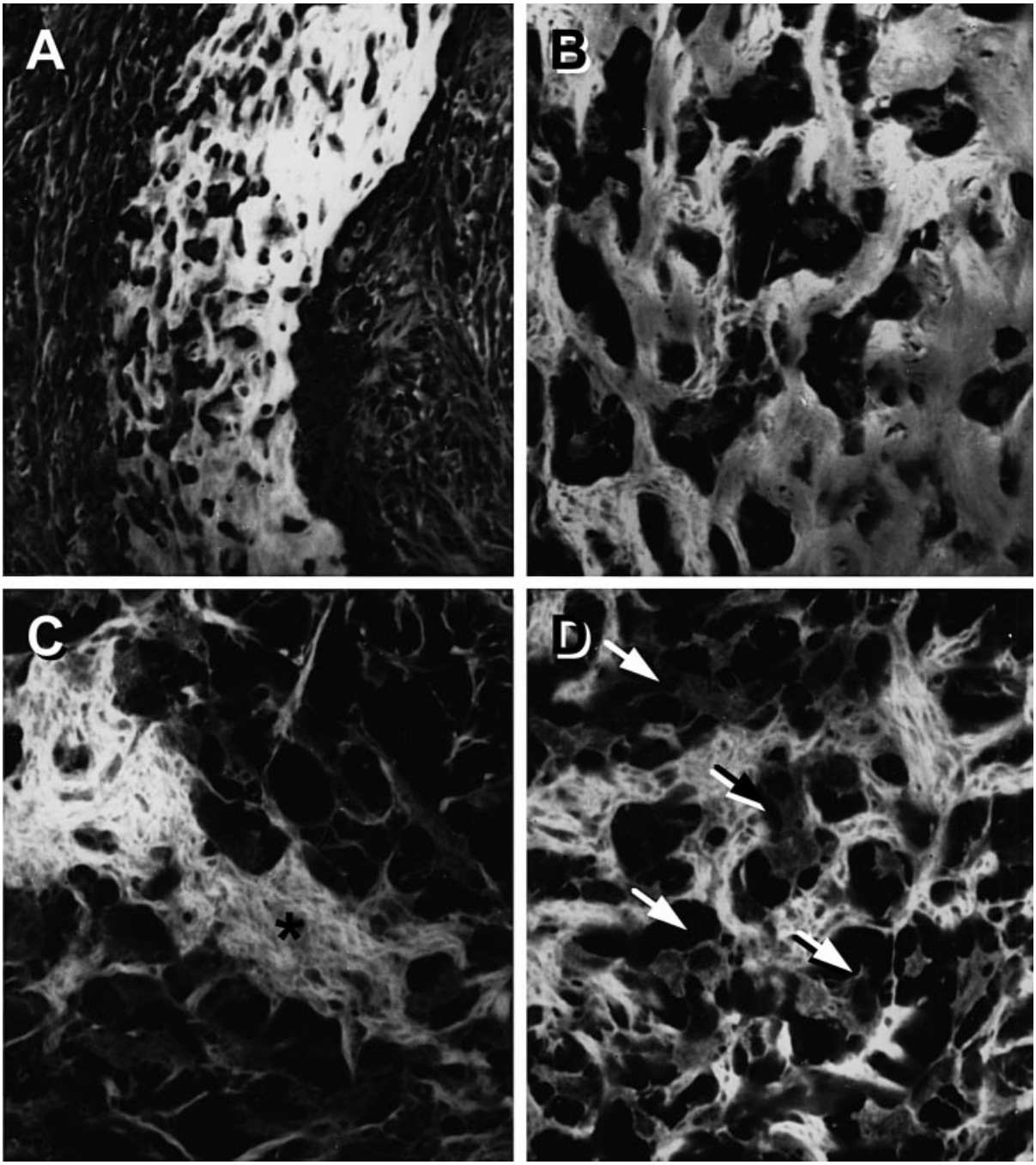


Fig. 5—Confocal images of 'hypercellular' FD. (A, B) Area of established trabecular bone. Note the complex systems of interconnected lacunar spaces harbouring multiple osteocyte-like cells and their polarization to one side (the formative side) of the bone trabeculae. (C, D) Area of initial membranous new bone formation. A forming osteoid appears as intensely fluorescent material in which individual collagen bundles can be resolved (C, asterisk). Osteoblastic cells associated with the forming osteoid are stellate in shape (D, arrows)

Our study also indicates that bone lesions other than FD commonly develop within MAS. Previous examples of some similar lesions in association with FD have been recorded[27–29] and yet this is the first instance in which these lesions were demonstrated in individuals with known mutations of the Gsα gene. On the other hand, we have demonstrated an R201H mutation in a patient with monostotic FD of the temporal bone, indicating, in agreement with two other recent reports,[11,12] that the occurrence of such mutations is not restricted to instances of clinically full-blown MAS in which the characteristic triad of endocrine disorders, polyostotic FD and skin pigmentation occurs. Isolated endocrine disorders in association with activating Gsα mutations or endocrine disorders associated with skin lesions in the absence of bone disease have been reported.[9,30–32] The recognition of mutation-positive, isolated, monostotic forms of FD in this and the two previous reports[11,12] thus extends the range of known, incomplete syndromic expression of the same basic disorders. Based on the

assumed post-zygotic occurrence of activating Gsα mutations and the somatic mosaic nature of MAS,[33] incomplete syndromic expressions, including monostotic FD, might represent the effect of mutations occurring at a later developmental stage and/or resulting in the survival of mutated cells in a localized region of the body. Segregating different clinical expressions of FD (monostotic, polyostotic, MAS) may thus not be warranted, based on a common underlying genetic defect, the prevalence of which in non-MAS cases of FD remains to be determined. Furthermore, the diversity of the histological expression of Gsα mutations in the bone environment, as shown here, suggests that besides monostotic forms of FD, other sporadic bone lesions may in principle be associated with the same mutations (e.g. aneurysmal bone cysts, giant cell 'reparative' granulomas, perhaps giant cell tumours of bone). Detecting the Gsα mutations in cells isolated directly from such non-FD lesions would be worthwhile. Reports of concurrent FD and aneurysmal bone cysts have been frequent and the concurrence and possible relationship of FD with other, admittedly unrelated bone lesions have also been occasionally noted.[34] Cemento-ossifying fibroma of the jaw bones and FD have been suggested to belong to a single continuous spectrum of lesions.[35] We have indeed observed one instance of combined cemento-ossifying fibroma and FD of hypercellular type in one patient with typical MAS which was not included in the present series due to the lack of mutational data.

Sharpey fibre systems and retracted/stellate osteoblasts were recently reported as characteristic features of FD of bone.[15] In this study, these features were characterized by confocal fluorescence microscopy. In addition to allowing for in-focus views through the whole section thickness, confocal fluorescence microscopy permits higher resolution than transmitted light microscopy. This gain of resolution was instrumental in visualizing subtle features of bone collagen texture and cell morphology and in showing their occurrence in all patterns of FD. Sharpey fibre bone and osteoblast retraction can therefore represent an important common denominator of the varied histological expressions of Gsα mutation-related FD. Sharpey fibre bone normally occurs at sites of insertion of tendons and ligaments, and at cranial sutures, i.e. at sites where tensile stresses are exerted over a forming bone surface.[36] In FD, stellate, branched osteoblasts are regularly associated with Sharpey fibres. *In vitro*, excess exogenous cAMP induces a comparable cell retraction in osteoblast-like cells.[15] It has been suggested that endogenous high levels of cAMP, generated by overstimulation of the adenylyl cyclase by the mutated Gsα, may represent the main determinant of osteoblast retracted/stellate shape in FD.[15] In this study, confocal microscopy demonstrated direct contacts between osteoblast cell processes and growing collagen (Sharpey) fibres at sites of bone formation. This suggests that excess endogenous cAMP may direct the deposition of abnormal (Sharpey fibre) collagen at a forming bone surface via the induction of osteoblast retraction and the generation of local tensile microstresses.

In conclusion, we have identified diverse site-specific histological patterns and common elementary lesions which characterize FD of bone in patients who have activating mutations of the α subunit of the G stimulatory protein gene. We believe that the recognition of such patterns and features is essential for the proper understanding, classification, and perhaps treatment of FD of bone, in the light of current approaches to its pathogenesis and clinical management.

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